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A Brilliant New Addition to the Fluorescent Probe Toolbox

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FLUORESCENT probes are critical for flow cytometry and fluorescent microscopy. Although new probes are developed and introduced commercially each year, there are only a selected few that are exceptional in what we commonly refer to as brightness. One such probe, phycoerythrin (PE), was first reported 30 years ago in 1982 for use in cell analysis as an antibody conjugate (1). The use of a second probe, allophycocyanin (APC), was reported a year later (2). It was not until 1998 that another probe of comparable brightness, the quantum dot, was reported. Unique from all others, the quantum dot is inorganic (3). Quantum dot probes have been especially useful because they are optimally excited by ultraviolet and violet light, a spectral region with limited coverage from other bright fluorescent probes. Now, a new probe (Brilliant Violet, BV) of a different class but of comparable brightness and excited by violet light has been developed as reported by Chattopadhyay et al. in this issue (page 456).

Although just 10 years ago flow cytometry experiments measuring more than six parameters or four "colors" were limited to relatively few advanced laboratories, this capability is now much more widely distributed, including even some resource limited settings. One reason for this increased accessibility is introduction of off-the-shelf and custom-modified instrumentation with greater capacity for multicolor detection and another reason is the growing commercial availability of reagents consisting of conjugates to less common fluorescent probes. The expansion of multicolor technology makes use of fluorescent probes that are excited at a variety of wavelengths, and to allow for this, instrumentation has been modified by adding additional lasers of different colors. In addition to the nearly universal blue and red lasers, a violet laser exciting at about 405 nm is now commonly included in many instruments (4).

Initially, there were very few fluorescent probes excited by the violet laser and they were dismal in terms of brightness. Soon, better probes and commercial conjugates became available although none rivaled the brightness of PE and APC. Therefore, the introduction of quantum dots caused a great amount of excitement not only because of their brightness, but also because of the selection of different colors all excited by the violet laser. Although quantum dots continue to be extremely useful, the initial enthusiasm has been tempered by some practical issues such as shelf life, sensitivity to heavy metal contaminants, tendency to form aggregates, and cross-laser compensation requirements that became more evident as they were included in multicolor panels (5). Thus, alternate violet-excited bright fluorescent probes are likely to be of benefit to many researchers (6).

The intrinsic brightness of a fluorescent compound is a function of the quantum yield and the molar extinction coefficient. The quantum yield is the probability of emitting a photon once a photon of light is absorbed and thus is a measure of the efficiency of energy conversion. High quantum yields are optimal and the quantum yield for BV is comparable to many other bright fluorochromes (Table 1). The molar extinc-

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Table 1. Quantum yield and extinction coefficient for commonly-used fluorescent probes

FLUOROCHROME	QUANTUM YIELD	MOLAR EXTINCTION COEFFICIENT[a]	EXCITATION/ABSORBANCE
Brilliant Violet	0.69	2,500,000	Violet (405 nm)
R-PE	0.82	1,960,000	Blue (496 nm)
APC	0.68	700,000	Red (650 nm)
Quantum Dot 655	≈0.3[b]	5,700,000	Violet (405 nm)
Quantum Dot 585	≈0.7[b]	2,200,000	Violet (405 nm)
AlexaFluor 660	0.37	132,000	Red (663 nm)
Fluorescein	0.5	86,000	Blue (488 nm)
AlexaFluor 488	0.92	71,000	Blue (495 nm)
Pacific Blue	0.78	46,000	Violet (405 nm)

[a]Measured at the indicated excitation/absorbance wavelength (cm^{-1} m^{-1}).
[b]Quantum yields generally increase for larger quantum dots (11).

tion coefficient is a measure of the probability of absorbing a photon of light at the wavelength of excitation and this is the measure that is exceptional for PE, APC, the quantum dots, and now BV. Other factors also affect the brightness of antibody conjugates such as the number of fluorophores conjugated to each antibody, but these intrinsic factors have a dominant effect (7).

The new fluorescent probe reported in this issue of Cytometry is a unique type of fluorescent compound with a fascinating pedigree stemming from a discovery that earned the Nobel Prize in chemistry in 2000. The discovery and development of conductive polymers has yielded many applications already in use in commercial products. The BV fluorescent probe resulted from chemical modifications to a polymer that yielded the appropriate excitation spectrum, water solubility, conjugation ability, and minimal nonspecific binding. Additional violet-excited fluorescent probes with differing emission spectra have been developed by creating tandem dyes of BV conjugated with more traditional fluorophores such as Cy3 (the BV570 tandem dye reported in this issue).

In this issue, Chattopadhyay and colleagues report on the use of these new dyes as antibody conjugates and as a streptavidin conjugate used with pMHCI multimers. The comparison of the BV421 conjugates with the Pacific Blue conjugates demonstrates their remarkable brightness, with the CD8 conjugate about 10-fold brighter as quantified by the stain index. Thus, the exceptional molar extinction coefficient translates into bright cell staining reagents. The authors also demonstrate suitable photostability and utility in fluorescence microscopy. Although only one tandem dye was tested, it is likely that a series of bright tandem dyes based on the Brilliant Violet backbone are forthcoming and will be a welcome addition to the ever-growing arsenal of fluorescent probes available to cytometrists.

Multicolor flow cytometry continues to be a cornerstone of immunological and cellular profiling. Although it is inevitable that new technologies will enter the field, such as nonfluorescent based flow cytometry (CyTOF) using mass spectrometry, where reagent labels are distinguished by mass and there is much less expertise required to assemble and optimize cell staining panels examining large numbers of markers (8), it is unlikely that they will replace fluorescent-based flow cytometry, at least in the near future. Thus, the art and science of creating staining panels remain important (9,10) and Brilliant Violet and its tandem dyes are useful additions to the toolbox of fluorescent probes. Indeed, there is a bright future for Brilliant Violet.

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